VOLUME 153 / No [redacted] OCTOBER 15, 1994

THE JOURNAL OF IMMUNOLOGY

THE AMERICAN ASSOCIATION OF IMMUNOLOGISTS

Applicants: David J. Pinsky
U.S. Serial No.: 09/374,586
Filed: August 13, 1999
Group Art Unit: 1633

Exhibit 25

HOST DEFENSE

The CD39 Lymphoid Cell Activation Antigen

Molecular Cloning and Structural Characterization

Charles R. Maliszewski,[1]* Guy J. T. Delespesse,† Michael A. Schoenborn,* Richard J. Armitage,* William C. Fanslow,* Toshiharu Nakajima,† Elizabeth Baker,‡ Grant R. Sutherland,‡ Kurt Poindexter,* Carl Birks,* Alan Alpert,* Della Friend,* Steven D. Gimpel,* and Richard B. Gayle III*

*Immunex Research and Development Corporation, Seattle, WA 98101; †University of Montreal, Notre-Dame Hospital Research Center, Montreal, Canada; ‡Center for Medical Genetics, Department of Cytogenetics & Molecular Genetics, Women's and Children's Hospital, North Adelaide, Australia

CD39, a 70- to 100-kDa molecule expressed primarily on activated lymphoid cells, was previously shown to mediate B cell homotypic adhesion when ligated with a subset of anti-CD39 mAbs. In the present study, we describe the cloning and molecular characterization of human and murine CD39. The nucleotide sequence of human CD39 includes an open reading frame encoding a putative 510 amino acid protein with six potential *N*-linked glycosylation sites, 11 Cys residues, and two potential transmembrane regions. Murine CD39 shares 75% amino acid sequence identity with human CD39 but fails to cross-react with anti-human CD39 mAbs. Although there were no significant similarities with other mammalian genes, considerable homology was found between CD39 and a guanosine diphosphatase from yeast. A series of mouse-human hybrid molecules was constructed to determine the general topology of CD39 and the location of a biologically functional epitope. These findings and supporting evidence from an anti-CD39 mAb-selected phage peptide display library indicate a likely model wherein a short intracellular N-terminus is followed by a large extracellular loop containing the epitope recognized by stimulatory anti-CD39 mAbs, and a short intracellular C terminus. The results demonstrate that CD39 is a novel cell surface glycoprotein with unusual structural characteristics. *The Journal of Immunology,* 1994, 153: 3574.

CD39 was originally defined as a B cell surface marker (1–3), but subsequent studies have defined a broader range of expression that includes subpopulations of activated NK cells, T cells, and certain T cell lines (4, 5). CD39 expression in lymphoid tissue is primarily limited to mantle zone and paracortical lymphocytes, macrophages, and dendritic cells and is generally absent from germinal centers (4). Endothelium and certain endothelial cell lines also express cell surface CD39 (4). The restricted expression of CD39 on activated lymphoid cells and in anatomical sites of ongoing B cell differentiation suggests that the molecule may play an important role in Ag-specific B cell responses. Studies demonstrate that CD39 ligation can result in signal transduction: treatment of $CD39^+$ B cell lines with certain anti-CD39 mAbs induces homotypic adhesion, a phenomenon that is energy dependent, involves tyrosine kinase activity, and is only partially mediated through LFA-1 (4, 6). The relevance of these findings to B cell growth and differentiation remains to be established.

CD39 has been immunoprecipitated as a 70- to 100-kDa protein that is heavily *N*-glycosylated (3, 4, 7); however, little else is known about the biochemical nature of this molecule. In the present study, we describe the cloning, expression, and molecular characterization of human CD39 as well as the identification of a murine CD39 homologue. The results indicate that CD39 is a unique cell surface molecule that contains two potential transmembrane regions and a hydrophobic segment within the extracellular region. In addition, the epitope recognized by

Received for publication April 11, 1994. Accepted for publication July 22, 1994.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Address correspondence and reprint requests to Dr. Charles R. Maliszewski, Immunex Research & Development Corporation, 51 University Street, Seattle, WA 98101.

0022-1767/94/$02.00

stimulatory anti-CD39 mAbs has been provisionally localized to two sites flanking the extracellular hydrophobic region.

Materials and Methods

Cells and reagents

The human MP-1 cell line is a spontaneously derived EBV-transformed B lymphoblastoid cell line (LCL)[2] grown from PBMC as previously described (8). The B73 mAb, a murine IgG1, was derived from BALB/c mice immunized with the RPMI 1788 cell line (9) and characterized as CD39-specific by flow cytometric analysis and immunoprecipitation/SDS-PAGE. The G28.10 mAb was the kind gift of Dr. Ed Clark, University of Washington (Seattle, WA), and the AC2 anti-CD39 mAb was purchased from AMAC, Inc. (Westbrook, ME).

Flow cytometry

For anti-CD39 binding experiments, MP-1 cells or transfected COS-7 or CV-1/EBNA cells were incubated with anti-CD39 mAbs B73, AC2, or G28.10 or murine IgG1 as an isotype control (10 μg/ml). After 30 min at 4°C, the cells were washed and incubated with a phycoerythrin-conjugated second Ab (affinity-purified goat anti-murine IgG; Tago, Inc., Burlingame, CA) for 30 min at 4°C, and then washed. Alternatively, a combination of biotinylated B73 mAb and phycoerythrin-conjugated streptavidin (SA-PE; Tago, Inc.) or biotinylated goat anti-human IgG Fc-specific Abs (Jackson ImmunoResearch, West Grove, PA), and SA-PE were used to stain cells transfected with CD39-Fc chimeras for CD39 and Fc expression, respectively. All Ab dilutions and washes were performed in PBS containing 10% FCS, 10% goat serum, 500 mM EDTA, and 0.02% sodium azide. Stained cells were analyzed on a FACScan flow cytometer by using a logarithmic fluorescence intensity scale. For fluorescence-activated cell sorting, transfected COS-7 cells were recovered from culture dishes with Versene and stained with Ab reagents as described above. The stained cells were sorted on an EPICS flow cytometer and 10,000 cells representing the top 0.1 to 0.3% brightest staining population were selected.

cDNA expression and immunoselection

Double-stranded cDNA was synthesized from polyadenylated RNA extracted from MP-1 cells and ligated into the mammalian expression vector pDC303 as previously described (8). Plasmid DNA from the resultant cDNA library, representing 500,000 individual clones, was purified by alkaline lysis and cesium chloride gradient centrifugation (10).

Three sequential rounds of transfection and immunoselection were performed as follows. In round I, plasmid DNA was transfected into subconfluent layers of COS-7 cells by using DEAE dextran followed by chloroquine treatment (11). $CD39^+$ cells were selected by panning on B73 mAb-coated plates according to established procedures (12). Episomal DNA was prepared from the panned cells, amplified in *Escherichia coli* and reintroduced into COS-7 cells (round II) by electroporation as follows. COS-7 cells were suspended in serum-free medium and placed into electroporation cuvettes (3×10^6/cuvette) along with 5 μg of plasmid DNA. Cells were electroporated by using 300 V at 500 μF. The cells were then placed into tissue culture plates with fresh, serum-containing medium. After incubating for 3 days, $CD39^+$ cells were selected by panning on B73-coated plates. Plasmid DNA recovered from round II-panned cells was electroporated into COS-7 cells (round III) and $CD39^+$ cells were selected by FACS as described above.

Plasmid DNA was recovered from the round III-sorted cells, transformed into *E. coli*, and divided into 10 pools containing 500 individual recombinant plasmids each. The final stage of cloning involved three successive rounds of transfection and screening with the use of a slide-based autoradiographic screening technique as previously described (8). Briefly, COS-7 cells were transfected in slide chambers and incubated for 3 days. Cells were then treated with ^{125}I-labeled B73 mAb, coated with photographic emulsion, and in situ autoradiography was performed. Positive pools were sequentially split until a pure clone was isolated. Results at each stage of screening were verified by flow cytometric analysis of transfectants.

Construction of a λgt10 library containing cDNA from the murine 70Z/3 pre-B cell line was previously described (13). The library (10^6 recombinant plaques) was screened for murine CD39 clones by using a radiolabeled cDNA probe representing the human CD39 coding region.

Sequence analysis

DNA sequencing was performed on an automated DNA sequencer (ABI, Foster City, CA). Sequence uniqueness and relatedness were determined by using the FASTA algorithm (14). Alignments between human and murine CD39 were performed by using GCG sequence analysis software. Identification of putative transmembrane regions used the criteria developed by Eisenberg et al. (15). The prediction protocol of Hartmann et al. was used to determine the spatial location of the amino terminus (16).

Immunoprecipitation and SDS-PAGE

MP-1 cells or transfected CV-1/EBNA cells (American Type Culture Collection, Rockville, MD; CRL 10478) were surface-labeled with biotin as previously described (17). Cells were then lysed for 5 min in extraction buffer consisting of PBS, Triton X-100 (1%), aprotinin (300 U/ml; Boehringer Mannheim, Indianapolis, IN) and EGTA (5 mM). After centrifugation at 12,000 × *g* for 10 min, 20 μl of the cell lysate was incubated with B73 mAb, M2 anti-CD40 mAb (Immunex R&D Corp.), or control murine IgG1 (10 μg/ml) for 1 h at 4°C. This mixture was incubated with 50 μl of protein A/G-agarose (Pierce, Rockford, IL) for an additional 30 min to allow the formation of a precipitable Ig matrix. The pellet was subsequently washed three times with 0.5% Nonidet P-40 in 10 mM Tris, 50 mM NaCl, pH 7.2, and once with PBS. Immunoprecipitated proteins were eluted with Laemmli buffer containing 200 mM DTT, boiled, and subjected to SDS-PAGE on a 10 to 20% gradient. After electrophoretic separation, the immunoprecipitated, biotinylated proteins were electrophoretically transferred to nitrocellulose filters. After transfer and washing in PBS, filters were blocked with PBS containing 1% nonfat dry milk for 1 h. The filters were washed twice in Tris-buffered saline (TBS), and then probed with streptavidin-horseradish peroxidase (Zymed, S. San Francisco, CA) in TBS containing 1% phosphatase-free BSA (Calbiochem, San Diego, CA) and 0.5% Triton X-100 for 1 h at room temperature. The filters were washed extensively in TBS and bound streptavidin was detected by using the ECL enhanced luminol chemiluminescent substrate for horseradish peroxidase (Amersham, Arlington Heights, IL). Emitted light was detected by exposure of the blot to Kodak X-OMAT film for exposure times of less than 10 min.

Hybridization techniques

Total or polyadenylated RNA from murine cells was subjected to electrophoresis in a formaldehyde/1.2% agarose gel, blotted by capillary flow onto a Nytran filter (Schleicher & Schuell, Keene, NH), and hybridized with a ^{32}P-labeled antisense riboprobe as previously described (18). The riboprobe consisted of nucleotides 1 to 1062 of the pHuCD39 (human CD39 expression plasmid) cDNA insert. The first wash was for 1 h in 2 × SSC at 50°C, then 20 min at 55°C in 0.5 × SSC, and finally 20 min at 63°C in 0.25 × SSC. Dried filters were exposed to Kodak X-OMAT AR film and developed.

Genomic mapping

The full-length cDNA insert in pHuCD39 was nick-translated with biotin-14-dATP. The cDNA probe was hybridized in situ at a final concentration of 15 ng/μl to metaphases from two normal male mice. The fluorescence in situ hybridization (FISH) method was modified from that previously described (19) in that chromosomes were stained before analysis with both propidium iodide (as counterstain) and DAPI (for chromosome identification).

Plasmid construction

Replacement of the CD39 C-terminal transmembrane region with the Fc region of human IgG1 was accomplished by using a unique *Pvu*II site

[2] Abbreviations used in this paper: LCL, lymphoblastoid cell line; FISH, fluorescence in situ hybridization; DAPI, 4'6-diamidino-2 phenylindole; SA-PE; phycoerythrin-conjugated streptavidin; TBS, Tris-buffered saline; Humu, chimeras of human CD39; Muhu, chimeras of murine CD39; pHuCD39, human CD39 expression plasmid.

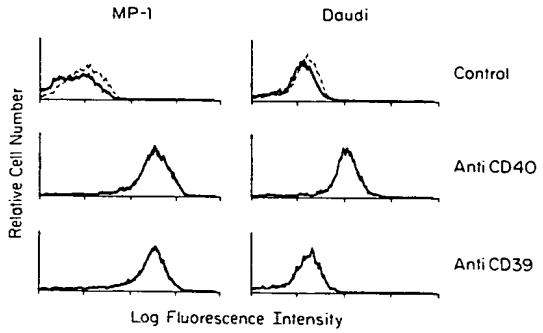

FIGURE 1. MP-1 cells express high levels of CD39. MP-1 or Daudi cells were stained with either anti-CD40 (G-28–5), anti-CD39 (B73), murine IgG1 isotype control (dashed line), or no mAb (solid line in control panels) followed by phycoerythrin-conjugated goat anti-mouse IgG. Cells were analyzed by flow cytometry as described in *Materials and Methods.*

(nucleotide 1480) that lies immediately upstream of the transmembrane region. An oligonucleotide linker containing *Pvu*II-*Bgl*II ends was used to reconstruct the transmembrane region proximal residues and to ligate to the Fc domain, essentially as previously described (20). Chimeras of human and murine CD39 (Humu and Muhu) were prepared in the pDC409 mammalian expression plasmid (21) by using *Sph*I and *Sst*I sites present in homologous positions in each gene. Humu1 was constructed by cleaving human CD39 and murine CD39 with *Sph*I and ligating the upstream region of human CD39 with the downstream portion of murine CD39. Muhu1 was constructed by ligating *Sph*I-generated upstream and downstream portions of murine and human CD39, respectively. Humu2 was constructed in a similar fashion to Humu1, by using *Sst*I-generated fragments. To generate Muhu2, an additional *Sst*I site present in murine CD39 was removed by site-directed mutagenesis, leaving a single *Sst*I site corresponding to the homologous site in the human CD39 and leaving the amino acid sequence intact. Thus, the Muhu2 construct was analogous to Muhu1, with the exception that *Sst*I-generated fragments were used.

Peptide display library

A filamentous phage display library (10^8 clones) containing random 15-mer amino acid sequences fused to the geneIII coat protein was constructed and screened by panning according to published protocols (22). Biotinylated mAb was bound to culture dishes precoated with goat anti-biotin Ab (Sigma Chemical Co., St. Louis, MO). The filamentous phage library was added to the Ab-coated dishes and incubated for 30 min at room temperature. After washing 10 times with TBS, specifically bound phage were eluted in the presence of excess nonbiotinylated mAb73. Eluted phage were amplified, the process was repeated through two more rounds, and recovered phage DNAs were sequenced. The peptide sequences derived from the phage library were aligned to regions in human CD39 by using the Multiple Alignment Construction and Analysis Workbench (23). This program aligns sequences with short regions of similarity, uses a variety of scoring tables, and offers more than one possible alignment for each sequence.

Results

Molecular cloning of human CD39

The B73 anti-human CD39 mAb was used to identify a $CD39^+$ cell source for cDNA library generation. Flow cytometric results in Figure 1 demonstrate high level binding of B73 mAb to MP-1, an EBV-transformed LCL, but not to Daudi, a Burkitt's lymphoma B cell line. In contrast, both cell types reacted with G28.5, an anti-CD40 mAb. These results are consistent with previous findings demonstrating that CD39 is expressed preferentially on LCL (1–3).

Poly-A^+ RNA was purified from MP-1 cells and used to construct a cDNA library in the pDC303 mammalian expression vector. Plasmid DNA representing the entire library of approximately 5×10^5 recombinants was transfected into COS-7 cells, and CD39-expressing transfectants were selected by panning on B73-coated plates. Plasmid DNA was recovered from sorted cells, amplified, and the entire process repeated through two more rounds (rounds II and III). Round III immunoselection was based upon flow cytometric sorting of $CD39^+$ cells. Recovered plasmid DNA was amplified, divided into 10 pools (500 recombinants per pool) and transfected into COS-7 cells grown on slide chambers. Transfected cells in this and subsequent rounds were screened by ^{125}I-labeled B73 mAb binding and slide autoradiography. Single clones were isolated from each of two B73-positive pools by two subsequent rounds of transfection/slide autoradiography. One of these clones, pHuCD39, was selected for further analysis.

Sequence analysis of CD39 cDNA

The nucleotide sequence of the pHuCD39 1.9-kb cDNA insert was determined (Fig. 2) and analysis of the corresponding amino acid sequence revealed the following features. An ATG codon marks the amino terminal methionine of a 510-amino acid protein with a predicted molecular mass of 57,000 Da. Human CD39 has two hydrophobic regions of approximately 20 amino acids, one near the amino terminus and the other near the carboxyl terminus, which could act as transmembrane regions according to the models of Eisenberg et al. (15). There is a third hydrophobic region at amino acids 347 to 367. In addition, there are six putative *N*-linked glycosylation sites and 11 cysteine residues.

Expression of recombinant human CD39 protein

COS-7 cells transfected with pHuCD39 expressed high levels of cell surface CD39 by flow cytometry (Fig. 3). By contrast, cells transfected with vector alone did not bind B73 mAb. The anti-CD39 mAbs AC2 and G28–10, which recognize the same epitope as B73, also bound to pHuCD39-transfected cells but not to control COS-7 cells.

Immunoprecipitation experiments were performed to further analyze recombinant CD39 expression. CV-1/EBNA cells transfected with either pHuCD39 or an irrelevant control plasmid were surface-labeled with biotin, and detergent lysates were prepared. Proteins were immunoprecipitated with B73 mAb and subjected to electrophoresis by SDS-PAGE. Specifically immunoprecipitated protein bands were identified by gel blotting and development with a streptavidin-horseradish peroxidase/luminol system. Results in Figure 4 indicate B73 binds to a protein of approximately 95 kDa from pHuCD39-transfected cells (*lane 5*). This protein band could be distinguished from a

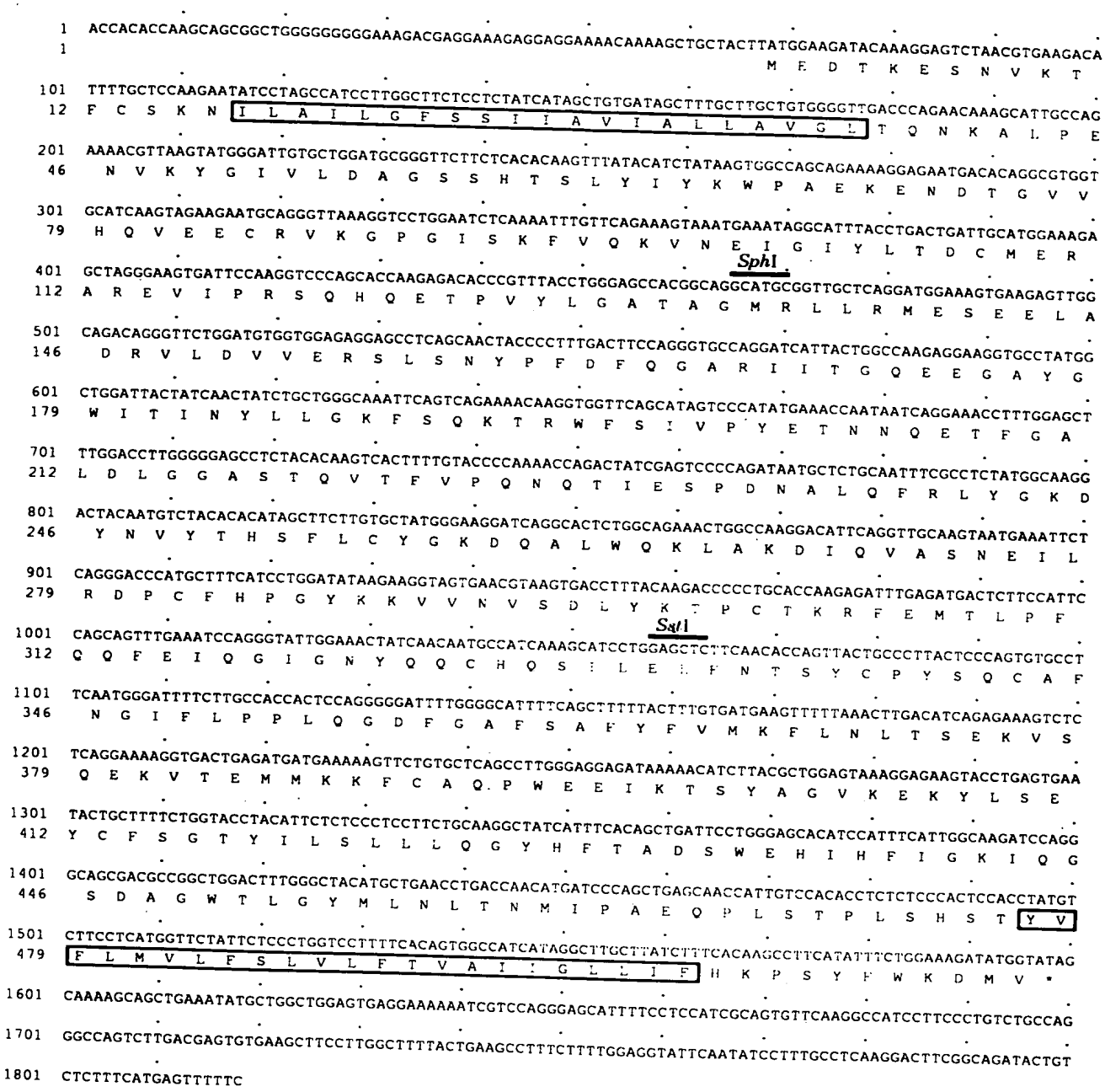

FIGURE 2. Human CD39 nucleotide and predicted amino acid sequences. Nucleotides are numbered beginning at the 5′ end, and amino acids are numbered beginning with the initiator methionine. Putative N- and C-terminal transmembrane regions are boxed. Sequences recognized by restriction enzymes *Sph*I and *Sst*I are indicated.

closely migrating band also present in *lane 4*, representing proteins nonspecifically immunoprecipitated from control-transfected cells. In comparison, MP-1 cell lysates were immunoprecipitated with either B73 or with anti-CD40 mAb, resulting in bands of approximately 100 kDa (*lane 2*) and 50 kDa (*lane 3*), respectively.

Chromosomal localization of human CD39

Twenty-five metaphases from a normal male were examined for fluorescent signal. Twenty-two of these metaphases showed signal on one or both chromatids of chromosome 10 in the region 10q23.1 to q24.1; 75% of this signal was at

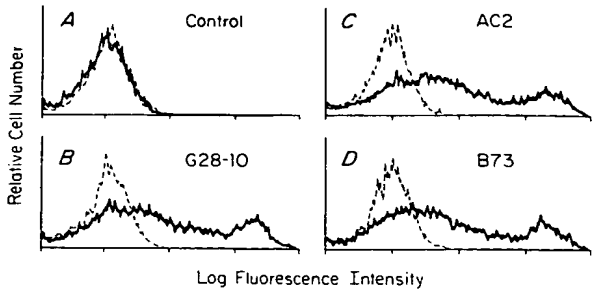

FIGURE 3. Cells transfected with pHuCD39 plasmid express surface CD39. Control plasmid (dashed line) and pHuCD39-transfected (solid line) COS-7 cells were stained with A, murine IgG1 isotype control; B, G-28–10 anti-CD39 mAb; C, AC2 anti-CD39 mAb; or D, B73 anti-CD39 mAb followed by phycoerythrin-conjugated goat anti-mouse IgG. Cells were analyzed by flow cytometry as described in *Materials and Methods.*

10q23.3 (Fig. 5). There was a total of 16 nonspecific background dots observed in these 25 metaphases. A similar result was obtained from the hybridization to 20 metaphases from a second normal male (data not shown).

Molecular cloning of murine CD39

A Northern blot containing Poly(A)$^+$ or total RNA from several murine cell sources was probed with a human CD39 antisense riboprobe (Fig. 6). Two activated T cell lines (7C2 and 7F9), a pre-B cell line (70Z/3), a mature B cell line (WEHI 231), a macrophage cell line (RAW 264.7), and primary bone marrow macrophages all expressed multiple forms of CD39 mRNA distinguishable from ribosomal RNA bands. On the basis of these results, the 70Z/3 pre-B cell line was chosen as a source of murine CD39 mRNA for library development and screening. More than 10^6 clones from a 70Z/3 cDNA library in λgt10 were screened by using a cDNA probe generated from the human CD39 sequence. Five clones that demonstrated significant binding to the probe were isolated and plaque-purified. These clones had inserts ranging in size from 910 to 2694 base pairs. One of these clones, 5B, contained an entire coding sequence and was selected for further analysis. An alignment of the deduced amino acid sequence in Figure 7 shows a large degree of sequence similarity between human CD39 and murine CD39 with 75% identity across the entire molecule. All 11 Cys residues and five of the putative *N*-linked glycosylation sites are conserved between human and murine CD39.

A search of the GenBank database identified a gene from *Saccharomyces cerevisiae* that displayed a high degree of similarity to CD39. As shown in Figure 7, the yeast guanosine diphosphatase (24) has about 27% sequence identity to both human and murine CD39 and almost 50% similarity. None of the potential *N*-linked glycosylation sites is conserved, but five consecutive Cys residues in the

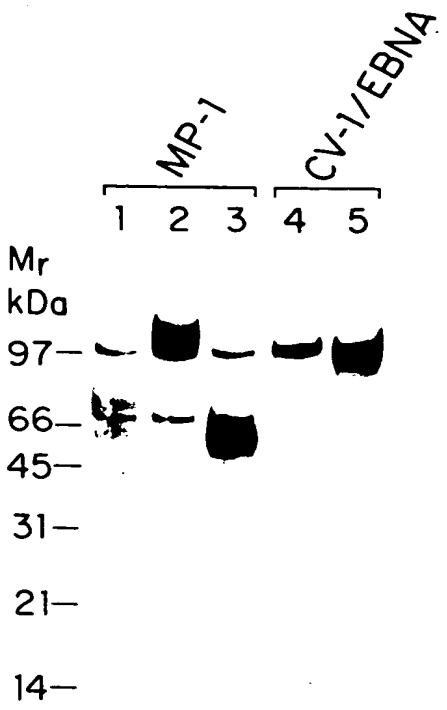

FIGURE 4. Anti-CD39 immunoprecipitates a 95-kDa molecule from transfected cells. MP-1 cells (*lanes 1-3*) or CV-1/EBNA cells transfected with control plasmid (*lane 4*) or pHuCD39 (*lane 5*) were biotin-labeled and detergent-lysed. Lysates were immunoprecipitated with isotype control mouse IgG1 (*lane 1*), B73 anti-CD39 mAb (*lanes 2, 4, 5*), or anti-CD40 mAb (*lane 3*) and analyzed by SDS-PAGE and Western blotting as described in *Materials and Methods.* Equivalent cell lysate numbers were loaded in every lane.

carboxyl half of CD39 are identically located in the yeast protein.

Epitope mapping and topologic analysis

We used the charge difference rule (15) to examine the 15 amino acid residues on either side of the amino terminal hydrophobic region of human CD39. This placed the amino terminus of CD39 on the inside of the cell membrane. To more fully delineate molecular topology, a number of expression plasmids were generated. First, the C-terminal transmembrane region and the sequences downstream were removed from human and murine CD39 and replaced with the Fc region of human IgG1. Binding of an anti-human IgG1 Ab to these fusion proteins would demonstrate the transport of the fusion protein to the cell surface. Binding of the B73 mAb would indicate the structure of the protein was not greatly perturbed by removal of the putative transmembrane region and addition of IgG1 Fc. COS cells were transfected with the resultant plasmids,

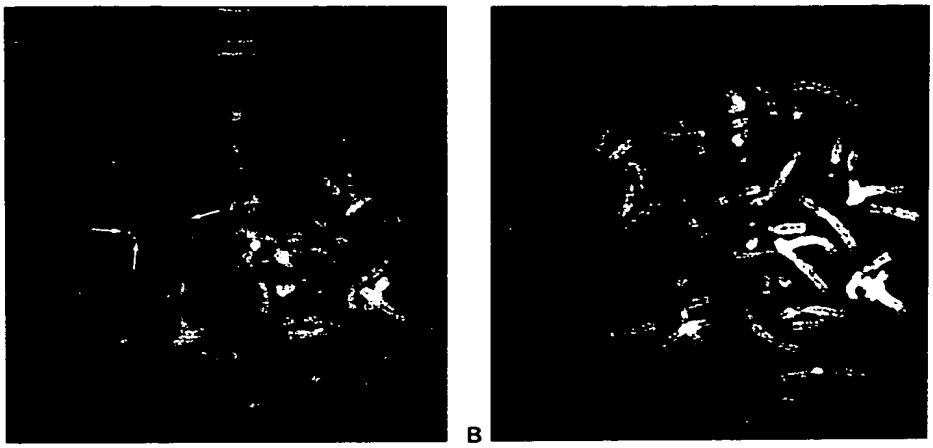

FIGURE 5. The human CD39 gene maps to chromosome 10. Metaphase showing FISH with pHuCD39 probe. *A*) Normal male chromosomes stained with propidium iodide. Hybridization sites are indicated by *arrows*. *B*) The same metaphase stained with DAPI for chromosome identification.

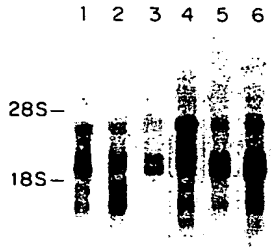

FIGURE 6. Northern blot analysis of murine CD39 mRNA expression. Total RNA from anti-CD3 activated T cell lines 7C2 (*lane 1*) and 7F9 (*lane 2*) and Poly(A)$^+$ RNA from 70Z/3 (*lane 3*), WEHI 231 (*lane 4*), RAW264.7 (*lane 5*) cell lines and bone marrow-derived macrophages (*lane 6*) were subjected to electrophoresis, blotted, and hybridized with a human CD39 riboprobe as described in *Materials and Methods*.

HuCD39-Fc and MuCD39-Fc, and subjected to flow cytometric analysis. Cells transfected with HuCD39-Fc retained the ability to react with the B73 mAb as well as with goat anti-human IgG1-Fc (Fig. 8). This result suggested that the portion of CD39 upstream of the C-terminal transmembrane region is normally extracellular and includes the epitope recognized by B73 mAb. Cells transfected with MuCD39-Fc bound anti-Fc Ab but failed to react with B73 mAb (Fig. 8), indicating that the CD39 epitope is species specific. Taken in conjunction with the postulated location of the amino terminus, a preliminary model would describe both the amino and carboxyl termini of CD39 as intracellular, and the majority of the protein would be extracellular. The central hydrophobic region could not fully transit the membrane in this model.

Additional structural analysis was performed by generating a series of human-murine CD39 chimeric plasmids and by using flow cytometry to localize the epitope for the B73 mAb. This strategy took advantage of 1) the presence of conserved restriction enzyme sites between the human and murine CD39 nucleotide sequences and 2) the finding that the murine B73 mAb fails to react with recombinant murine CD39 (Fig. 8). All of the chimeras were constructed as Fc fusion proteins, thus allowing detection of recombinant protein at the cell surface in the absence of the CD39 epitope. The chimeric proteins are schematically represented in Figure 9 along with the percentages of transfected cells expressing the chimeric proteins. These values are determined on the basis of flow cytometric analysis, as depicted in Figure 8. All four of the recombinant chimeric proteins reacted with anti-Fc Ab. Muhu1 reacted with anti-CD39, whereas Humu1 did not, indicating that the epitope recognized by B73 mAb lies downstream of residue 134 (Fig. 9). Neither Humu2 nor Muhu2 reacted with anti-CD39, suggesting that the epitope is not wholly contained in the region between amino acid residues 134 and 330, or in the region between residues 330 and 476 (Fig. 9). Either the chimeric proteins have greatly altered structure, which is unlikely because of the homologous nature of the mouse and human protein sequences, or the epitope is composed of noncontiguous regions of CD39.

Additional evidence for the nonlinear nature of the CD39 epitope was obtained by using a peptide display system with the use of filamentous phage. A phage library containing random 15-mer amino acid sequences fused to geneIII was created and screened for the ability to bind to B73 mAb. Several sequences were obtained and aligned with the human CD39 sequence. Two regions of similarity were identified (Fig. 10), one mapping near residue 155, between the *Sph*I and *Sst*I sites, and a second region downstream from the *Sst*I site, centered around residue 450. This finding was consistent with the results of the chimera analysis (Fig. 9) and suggested the existence of a split epitope.

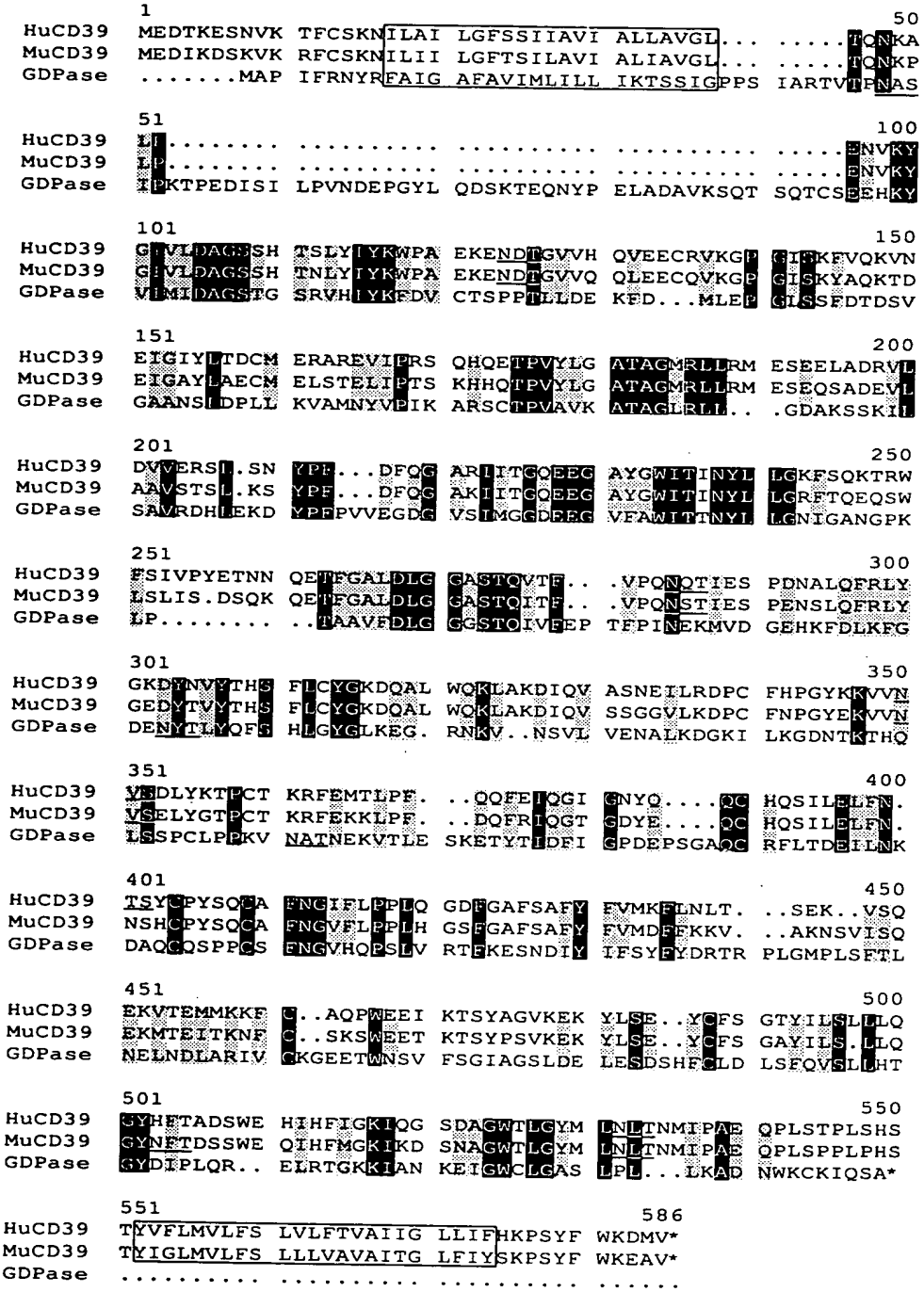

FIGURE 7. Alignment of human CD39 and murine CD39 amino acid sequences with yeast guanosine phosphatase sequence. Horizontal bars represent potential N-glycosylation sites; stars indicate shared cysteine residues; and boxed areas represent potential transmembrane regions. Identical residues between all three proteins are shaded in black; similar residues are shaded in gray. Gaps have been inserted where necessary (dots) to maintain sequence alignment.

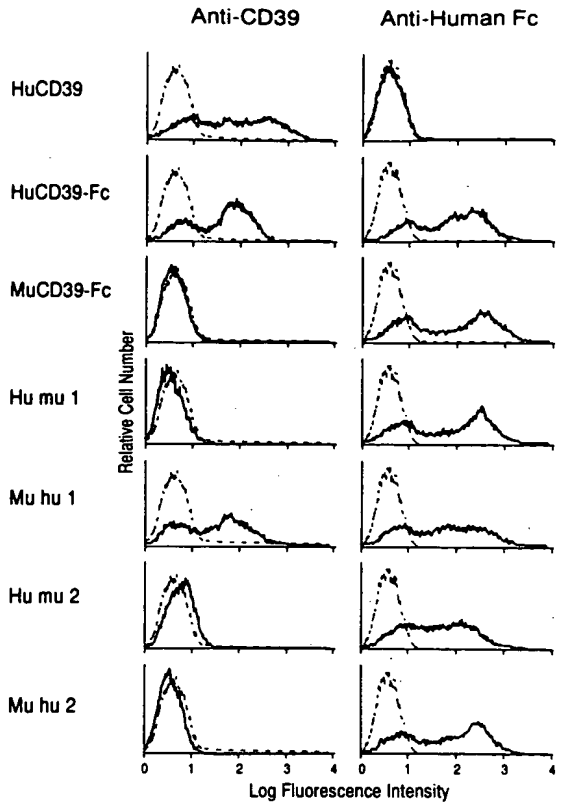

FIGURE 8. Flow cytometric analysis of cells expressing human and murine CD39-Fc molecules. COS were transfected with plasmids encoding full-length human CD39 cDNA (HuCD39), human or murine CD39 with the carboxy-terminal transmembrane region replaced by human IgG1-Fc (HuCD39-Fc and MuCD39-Fc), or human-murine CD39 chimeras. Transfected cells were incubated with either biotinylated B73 mAb (anti-CD39) or anti-human Fc followed by SA-PE, and developed for flow cytometric analysis as described in *Materials and Methods*.

Discussion

This paper describes the cloning and molecular characterization of the CD39 activation Ag. An immunoselection-based technique with an anti-CD39 mAb was used to isolate from an EBV-positive cell line cDNA library a clone encoding full-length human CD39. Cells transfected with the pHuCD39 expression plasmid reacted with three anti-CD39 mAbs directed against the same epitope (Fig. 3). The B73 mAb immunoprecipitated a protein of approximately 95 kDa from transfected cells, consistent with the reported m.w. of native CD39 (3, 4, 7). Moreover, the predicted peptide molecular mass (57 kDa) and the presence of six potential *N*-glycosylation sites on recombinant CD39 is consistent with the observed molecular mass (54 kDa) of native CD39 after enzymatic removal of *N*-linked sugars (4). These observations indicate that the recombinant molecule is likely to be CD39. However, given the unavailability of anti-CD39 mAbs directed against other epitopes, further confirmation of identity must await ongoing attempts to obtain amino acid sequence of native CD39 and the development of mAbs against the recombinant molecule.

In consideration of the possibility that the cloned molecule is not itself CD39 but actually is required for expression of CD39 by transfected cells, we transfected murine macrophages with the human CD39 expression plasmid. The B73 anti-human CD39 mAb bound to the transfected murine cells but not to mock-transfected macrophages (our manuscript in preparation). Because this mAb does not cross-react with murine CD39, we concluded that de novo synthesis of recombinant human CD39 occurred in these cells. In addition, we have determined by fluorescence microscopy that untransfected or mock-transfected COS cells do not express intracellular CD39 (data not shown), indicating that the recombinant protein is not simply allowing cell surface expression of constitutively synthesized, native CD39.

The structure of CD39 in the cell membrane was investigated by using several approaches. Protein sequence analysis identified three hydrophobic regions. The amino- and carboxyl-terminal regions are sufficiently hydrophobic to be transmembrane helices; by the same model, the central hydrophobic region would not be capable of fully penetrating the helix on its own but could form a transmembrane helix if the other two helices helped stabilize it (16). The topology of the molecule in the membrane would depend upon the spatial location of the amino and carboxyl termini. The charge distribution at the amino terminus places it on the cytoplasmic side of the membrane. Constructions that remove the C-terminal hydrophobic region and replace it with human Fc demonstrate the sequences proximal to this region are outside the cell, thus placing the C terminus inside. The human Fc region is undetectable when cells are transfected with a plasmid construct consisting of full-length human CD39 ligated to human IgG1 Fc (data not shown). The presence of both the amino and carboxyl termini in the cytoplasm implies that the central hydrophobic region either does not fully penetrate the membrane or is not associated with the membrane at all.

Chimeras between murine CD39, which does not react with the B73 mAb, and human CD39 were constructed to localize the CD39 epitope and further define extracellular region structure. From the flow cytometric data (Figs. 8 and 9), it would seem that the epitope recognized by B73 is located downstream from the location of the *Sph*I site that encompasses amino acids 134 to 136 (Fig. 9). It would also seem that the epitope is not linear, that is, human CD39 sequences upstream and downstream of residue 330 are required for B73 mAb binding. This conclusion is supported by results from panning experiments with the phage display system. The two regions of human CD39 identified by the 16-phage sequences fall within the candidate

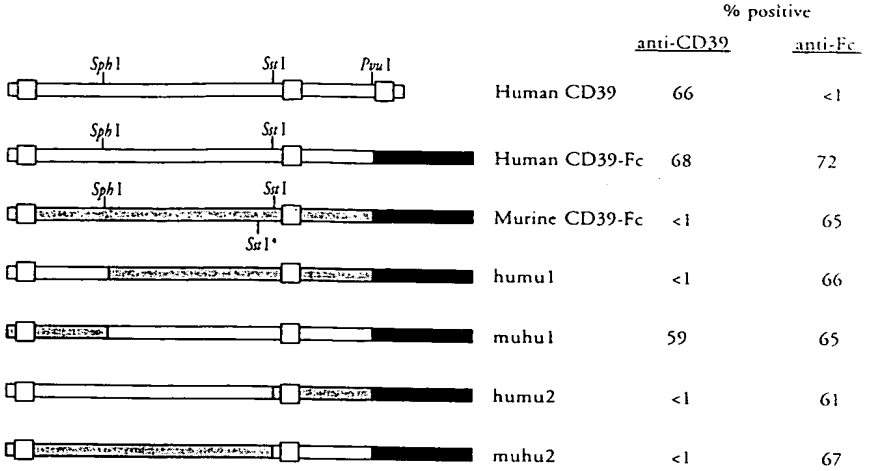

FIGURE 9. Cell surface expression of human-murine CD39 chimeras. The murine and human regions of CD39 for each construction are schematically represented. Boxes indicate locations of hydrophobic regions. COS cells transfected with plasmids containing the depicted cDNA inserts were analyzed by flow cytometry for cell surface expression of 1) the human CD39 epitope recognized by B73 mAb or 2) human IgG1 Fc recognized by goat anti-human Ab as described in *Materials and Methods*. The percentages of cells positively staining with each Ab are presented next to each plasmid construct.

A

```
          140                 160
 HuCD39   ESEELADRVL DVVERSLSNY PFDFQGARII
 MuCD39   ESEQSADEVL AAVSTSLKSY PFDFQGAKII
11-D2010  ....SQDRLL NNSGRSLSQ. ..........
13-D2010  .........V DALVRSTSAF HAPP......
 3-D1360  .......... GNHSRSYTIY KTLWP.....
 9-D1360  .......... ..LYRSPLLY PTSLLAG...
15-D2010  ...ESSHRWP DPESRLAS.. ..........
 4-D1360  .......... FAWSRYSSSP NVMFW.....
 1-D1360  ........WK FEYRSSASIY WNP.......
18-D2010  ...MIKRDSG.SHTILSLS.. ..........
17-D2010  ........RH QGASLIQNSY .RDF......
24-D2010  .....  WQS MNDNLATSNN AQ .......
14-D2010  .......... PINRQTYGSI PSQFE.....
```

B

```
          431                           460
 HuCD39   FTADSWEHIH FIGKIQGS.D AGWTLGYMLN LTNMIPAEQP
 MuCD39   FTDSSWEQIH FMGKIKDS.N AGWTLGYMLN LTKMIPGEQP
 8-D1360  .......... ....HRTQVN AGLTTITLR. ..........
23-D2010  .......... .......SAN AGFTRLAIPV AW........
 2-D1360  .......... .....SDTLS AGLRRT.LSA L.........
22-D2010  .......... .....SDTLS AGLRRT.LSA L.........
12-D2010  .......... .LSK KATPD ETWVAQI... ..........
```

FIGURE 10. Alignment of phage-derived amino acid sequences with regions of CD39. Sixteen sequences from phage panned with B73 mAb were analyzed and aligned with human CD39 by using the Multiple Sequence Alignment Construction and Analysis Workbench (23). *A*) region of CD39 with highest similarity to phage-derived sequences. *B*) region of second highest similarity.

epitope regions as identified by chimera analysis. The first region, centered around an Arg-Ser-Leu-Ser motif at residue 155, yielded the best alignment between CD39 and some phage sequences. In one phage sequence, 11-D2010, 11 of the 15 residues display a high degree of similarity to CD39. Several residues in this region differ between human and murine CD39. The second region, near residue 451, does not display quite the degree of similarity as the first site; nonetheless, several residues in the phage sequences align very well with amino acids within this region of CD39. Further mutagenesis of human CD39, concentrating on these two regions, will be necessary to confirm the split epitope hypothesis.

The similarity between yeast guanosine diphosphatase and CD39 is intriguing. Guanosine diphosphatase is a type II membrane protein with a cytoplasmic amino terminus (24); however, there is no carboxyl-terminal hydrophobic region analogous to that found in CD39, as the sequence of guanine diphosphatase ends a few residues upstream of this site. Nevertheless, sequence similarity extends across the length of the guanosine diphosphatase molecule (Fig. 7). The high degree of similarity between CD39 and guanosine diphosphatase suggests that these proteins form, at the very least, a structurally related family of molecules. Guanosine diphosphatase is found in the lumen of the Golgi apparatus and is involved in glycosylation of proteins and sphingolipids, catalyzing the removal of a phosphate from a GDP after sugar transfer (25). Whether CD39 has an associated enzymatic activity remains to be determined. Nonetheless, enzymatic activities have been attributed to other lymphoid cell surface molecules, notably CD38, which catalyzes the synthesis of cyclic ADP-ribose that can enhance B cell proliferation (26).

Previous reports have demonstrated that certain mAbs to CD39, including AC2 and G-28–10, can trigger homotypic adhesion (4, 6). This function is also displayed by several other B cell surface molecules, including CD19. CD20, CD40, and MHC class II (6). Although additional activities have been attributed to these molecules, the relevance of homotypic adhesion to B cell function remains unclear as does the means by which signals for these different activities are transduced. In fact, there are no obvious correlations between structure and the ability of each of these molecules to signal homotypic adhesion. It is possible, however, that this function is mediated by way of interactions with one or more additional molecules at the cell surface. CD39, lacking both a sizable intracellular region and obvious intracellular catalytic sites, would seem unlikely to directly transmit biologic signals. Additional experimentation will be required

to define the mechanism(s) by which CD39 mediates biologic activity (i.e., receptor vs enzyme).

Acknowledgments

The authors thank Guy Caspary and the Transfection Group for technical assistance; Terry Farrah for sequence alignment analysis; Drs. Steven Ziegler, John Sims, and Kim Campbell for critical review of the manuscript; and Anne C. Bannister for editorial review.

References

1. Rowe, M., J. E. Hildreth, A. B. Rickinson, and M. A. Epstein. 1982. Monoclonal antibodies to Epstein-Barr virus-induced, transformation-associated cell surface antigens: binding patterns and effect upon virus-specific T-cell cytotoxicity. *Int. J. Cancer 29:373.*
2. Ling, N. R., D. Hardie, J. Lowe, G. D. Johnson, M. Khan, and I. C. MacLennan. 1989. A phenotypic study of cells from Burkitt lymphoma and EBV-B-lymphoblastoid lines and their relationship to cells in normal lymphoid tissues. *Int. J. Cancer 43:112.*
3. Dörken, B., P. Möller, A. Pezzutto, R. Schwartz-Albiez, and G. Moldenhauer. 1989. B-cell antigens: CD39. In *Leucocyte Typing IV.* W. Knapp, B. Dörken, W. R. Gilks, E. P. Rieber, R. E. Schmidt, H. Stein, and A. E. G. K. von dem Borne, eds. Oxford University Press, Oxford, p. 90.
4. Kansas, G. S., G. S. Wood, and T. F. Tedder. 1991. Expression, distribution, and biochemistry of human CD39: role in activation-associated homotypic adhesion of lymphocytes. *J. Immunol. 146: 2235.*
5. Gouttefangeas, C., I. Mansur, M. Schmid, H. Dastot, C. Gelin, G. Mahouy, L. Boumsell, and A. Bensussan. 1992. The CD39 molecule defines distinct cytotoxic subsets within alloactivated human CD8-positive cells. *Eur. J. Immunol. 22:2681.*
6. Kansas, G. S., and T. F. Tedder. 1991. Transmembrane signals generated through MHC class II, CD19, CD20, CD39, and CD40 antigens induce leukocyte function-associated antigen-1-dependent and independent adhesion in human B cells through a tyrosine kinase-dependent pathway. *J. Immunol. 147:4094.*
7. Ledbetter, J. A., T. T. Tsu, N. A. Norris, and E. A. Clark. 1987. Characterization of a new 80–85 kDa B cell cluster (CD39) with monoclonal antibodies G28–8 and G28–10. In *Leukocyte Typing III.* A. J. McMichael, ed. Oxford University Press, New York, p. 349.
8. Goodwin, R. G., M. R. Alderson, C. A. Smith, R. J. Armitage, T. VandenBos, R. Jerzy, T. W. Tough, M. A. Schoenborn, T. Davis-Smith, K. Hennen, B. Falk, D. Cosman, E. Baker, G. R. Sutherland, K. H. Grabstein, T. Farrah, J. G. Giri, and M. P. Beckmann. 1993. Molecular and biological characterization of a ligand for CD27 defines a new family of cytokines with homology to tumor necrosis factor. *Cell 73:447.*
9. Rector, E., T. Nakajima, C. Rocha, D. Duncan, D. Lestourgeon, R. S. Mitchell, J. Fischer, A. H. Schon, and G. Delespesse. 1985. Detection and characterization of monoclonal antibodies specific to IgE receptors on human lymphocytes by flow cytometry. *Immunology 55:481.*
10. Sambrook, J., E. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual.* Cold Spring Harbor Press, Cold Spring Harbor.
11. Cosman, D., D. P. Cerretti, A. Larsen, L. Park, C. March, S. Dower, S. Gillis, and D. Urdal. 1984. Cloning, sequence and expression of human interleukin-2 receptor. *Nature 312:768.*
12. Seed, B., and A. Aruffo. 1987. Molecular cloning of the CD2 antigen, the T-cell erythrocyte receptor, by a rapid immunoselection procedure. *Proc. Natl. Acad. Sci. USA 84:3365.*
13. McMahan, C. J., J. L. Slack, B. Mosley, D. Cosman, S. D. Lupton, L. L. Brunton, C. E. Grubin, J. M. Wignall, N. A. Jenkins, C. I. Brannan, N. G. Copeland, K. Huebner, C. M. Croce, L. A. Cannizzarro, D. Benjamin, S. K. Dower, M. K. Spriggs, and J. E. Sims. 1991. A novel IL-1 receptor, cloned from B cells by mammalian expression, is expressed in many cell types. *EMBO J. 10:2821.*
14. Pearson, W. R., and D. J. Lipman. 1988. Improved tools for biological sequence comparison. *Proc. Natl. Acad. Sci. USA 85:2444.*
15. Eisenberg, D., E. Schwarz, M. Komaromy, and R. Wall. 1984. Analysis of membrane and surface protein sequences with the hydrophobic moment plot. *J. Mol. Biol. 179:125.*
16. Hartmann, E., T. A. Rapoport, and H. F. Lodish. 1989. Predicting the orientation of eukaryotic membrane-spanning proteins. *Proc. Natl. Acad. Sci. USA 86:5786.*
17. Fanslow, W. C., K. N. Clifford, J. D. Zappone, M. R. Alderson, and R. J. Armitage. 1993. CD40 mAbs M2 and M3 inhibit CD40L binding and function. *Leukocyte Typing V. In press.*
18. Maliszewski, C. R., C. J. March, M. A. Schoenborn, S. Gimpel, and L. Shen. 1990. Expression cloning of a human Fc receptor for IgA. *J. Exp. Med. 172:1665.*
19. Callen, D. F., E. Baker, H. J. Eyre, J. E. Chernos, J. A. Bell, and G. R. Sutherland. 1990. Reassessment of two apparent deletions of chromosome 16p to an ins(11;16) and a t(1;16) by chromosome painting. *Ann. Genet. 33:219.*
20. Fanslow, W. C., D. Anderson, K. H. Grabstein, E. A. Clark, D. Cosman, and R. J. Armitage. 1992. Soluble forms of CD40 inhibit biological responses of human B cells. *J. Immunol. 149:655.*
21. Smith, C. A., H.-J. Gruss, T. Davis, D. Anderson, T. Farrah, E. Baker, G. R. Sutherland, C. I. Brannan, N. G. Copeland, N. A. Jenkins, K. H. Grabstein, B. Gliniak, I. B. McAlister, W. Fanslow, M. Alderson, B. Falk, S. Gimpel, S. Gillis, W. S. Din, R. G. Goodwin, and R. J. Armitage. 1993. CD30 antigen, a marker for Hodgkin's lymphoma, is a receptor whose ligand defines an emerging family of cytokines with homology to TNF. *Cell 73:1349.*
22. Smith, G. P., and J. K. Scott. 1993. Libraries of peptides and proteins displayed on filamentous phage. *Methods Enzymol. 217:228.*
23. Schuler, G. D., S. F. Altschul, and D. J. Lipman. 1991. A workbench for multiple alignment construction and analysis. *Proteins 9:180.*
24. Abeijon, C., K. Yanagisawa, E. C. Mandon, A. Hausler, K. Moremen, C. B. Hirschberg, and P. W. Robbins. 1993. Guanosine diphosphatase is required for protein and sphingolipid glycosylation in the Golgi lumen of *Saccharomyces cerevisiae. J. Cell. Biol. 122:307.*
25. Abeijon, C., P. Orlean, P. W. Robbins, and C. B. Hirschberg. 1989. Topography of glycosylation in yeast: characterization of GDPmannose transport and lumenal guanosine diphosphatase activities in Golgi-like vesicles. *Proc. Natl. Acad. Sci. USA 86:6935.*
26. Howard, M., J. C. Grimaldi, J. F. Bazan, F. E. Lund, L. Santos-Argumedo, R. M. Parkhouse, T. F. Walseth, and H. C. Lee. 1993. Formation and hydrolysis of cyclic ADP-ribose catalyzed by lymphocyte antigen CD38. *Science 262:1056.*